Credentiaing a Preclinical Mouse Model of Alveolar Rhabdomyosarcoma

Koichi Nishijo, Qing-Rong Chen, Lei Zhang, Amanda T. McCleish, Andrea Rodriguez, Min Jung Cho, Suresh I. Prajapati, Jonathan A.L. Gelfond, Gary B. Chisholm, Joel E. Michalek, Bruce J. Aronow, Frederic G. Barr, R. Lor Randall, Marc Ladanyi, Stephen J. Qualman, Brian P. Rubin, Robin D. LeGallo, Chiayeng Wang, Javed Khan, and Charles Keller 1,2,4

Abstract

The highly aggressive muscle cancer alveolar rhabdomyosarcoma (ARMS) is one of the most common soft tissue sarcoma of childhood, yet the outcome for the unresectable and metastatic disease is dismal and unchanged for nearly three decades. To better understand the pathogenesis of this disease and to facilitate novel preclinical approaches, we previously developed a conditional mouse model of ARMS by faithful recapitulating the genetic events observed in the human disease, i.e., activation of Pax3:Fkhr fusion gene with either p53 or Cdkn2a inactivation. In this report, we show that this model recapitulates the immunohistochemical profile and the rapid progression of the human disease. We show that Pax3:Fkhr expression increases during late preneoplasia but tumor cells undergoing metastasis are under apparent selection for Pax3:Fkhr expression. At a whole-genome level, a cross-species gene set enrichment analysis and metagene projection study showed that our mouse model is most similar to human ARMS when compared with other pediatric cancers. We have defined an expression profile conserved between mouse and human ARMS, as well as a Pax3:Fkhr signature, including the target gene, SKP2. We further identified 7 “druggable” kinases overexpressed across species. The data affirm the accuracy of this genetically engineered mouse model. [Cancer Res 2009;69(7):2902–11]

Introduction

Rhabdomyosarcoma is the most common soft tissue tumor in childhood (1). Pediatric rhabdomyosarcoma can be divided into two major subtypes, embryonal rhabdomyosarcoma (ERMS) and alveolar rhabdomyosarcoma (ARMS; ref. 1). ERMS comprises 50% to 60% of all rhabdomyosarcoma cases and typically manifests a favorable outcome, whereas 20% to 30% of rhabdomyosarcoma are of the more aggressive alveolar subtype that is associated with frequent metastasis at the time of initial diagnosis (2). The development of more effective therapies in ARMS, however, has been hampered by a lack of knowledge about basic molecular mechanisms of tumor development. Cytogenetic and molecular studies show that 70% to 85% of ARMS have balanced chromosomal translocations of t(2;13) or t(1;13), which lead to the formation of chimeric transcription factors consisting of the NH2 terminal regions of Pax3 or Pax7 fused to the COOH terminal region of Fkhr (3). Pax3:Fkhr-positive ARMS is more aggressive than Pax7:Fkhr-positive or fusion-negative ARMS, thus, Pax3:Fkhr-positive ARMS represents the most clinically intractable subset of ARMS (4).

We previously generated a conditional knock-in allele of Pax3:Fkhr in Pax3 locus and established a mouse model of ARMS by simultaneously activating Pax3:Fkhr expression and inactivating p53 or Cdkn2a in Myf6-expressing maturing myofibers (5–7). In the current study, we show that this model authentically recapitulates the natural history, histologic features, and genetic features of the human disease, and we show the utility of this model in understanding the aspects of disease progression and therapeutic target identification.

Materials and Methods

Mice. The conditional models of ARMS have been previously described (5). At necropsy, animals were sacrificed by CO2 asphyxiation in accordance with an approved Institutional Animal Care and Use Committee protocol. Characteristics of mouse tumor and skeletal muscle samples used for microarray and quantitative reverse transcription–PCR (qRT-PCR) are described in Supplementary Tables S1 and S2.

Real-time RT-PCR. qRT-PCR analyses were performed by a Taqman assay for mouse Pax3:Fkhr expression or by SYBR Green assay (PE Applied Biosystems) for other genes of interest. Primer and probe sequences are described in Supplementary Tables S1 and S2.

Gene expression analysis. Gene expression analysis was performed using Affymetrix Mouse 430A arrays (Affymetrix). Original CEL files of the mouse ARMS are uploaded in the Gene Expression Omnibus site. For human tumors, published data sets of rhabdomyosarcomas (8, 9, juvenile and old skeletal muscles (10), Duchene muscular dystrophy (11), and a series of mesenchymal tumors (12, 13) and pediatric malignancies (14) were used (Supplementary Table S5). For mouse tumors, published data sets of...
osteosarcoma (15) and medulloblastoma (16) were used. Methods of microarray analysis, including gene set enrichment analysis (GSEA) and metagene analysis, are described in Supplementary Materials and Methods.

**CAT and luciferase reporter assays.** CAT constructs containing SKP2 promoter were described previously (17). The 220-bp genomic fragment 49-kb 3′ to Ski2 gene was inserted into pGL4.24 vector (Promega). Reporter plasmids were cotransfected with Pax3:Fkh and p53 into NIH3T3 cells or p53-deficient mouse embryonic fibroblasts (MEF).

**Western blotting.** Western blotting was performed as previously described (18). Antibodies against p27Kip1 (C-19), Ski2 (H-435), and Fkh (C-20) were from Santa Cruz. Pax3 antibody (ab-2) was from Geneka. α-Tubulin antibody was from Oncogene.

**Results**

Biallelic activation of Pax3:Fkh and disruption of p53 or Cdkn2a are necessary for high penetrance of ARMS. The mean latency of ARMS development was 110 days with 100% penetrance of ARMS when biallelic activation of conditional Pax3:Fkh allele was combined with homozygous deletion of conditional p53 allele (Fig. 1A). However, when the mice had homozygous Pax3:Fkh and heterozygous p53 mutant alleles or heterozygous Pax3:Fkh and homozygous p53 mutant allele combinations, tumor incidence was significantly lower than that of double homozygous alleles (P < 0.001), indicating a mutation dosage effect. As previously described, activation of Pax3:Fkh was necessary but not sufficient for ARMS development (5). When Pax3:Fkh allele was combined with conditional Cdkn2a mutation, mice still required biallelic activation of both mutations to develop ARMS at 100% penetrance (Fig. 1B). There was no significant difference in ARMS development between Pax3:Fkh-p53 mice and Pax3:Fkh-Cdkn2a mice (Fig. 1C). All ARMS cases were diagnosed by a qualified pathologist based upon histology, as well as MyoD and Myogenin immunohistochemistry (Fig. 1D). To determine the relative contribution of p53 mutation to the development of ARMS, Myf6Cre/Wt Pax3P3Fm/P3Fmp53F2-10/F2-10 mice were compared with Myf6Cre/Wt Pax3WT/P53p53F2-10/F2-10 (Supplementary Fig. S1). Myf6Cre/Wt Pax3WT/P53p53F2-10/F2-10 tumors developed pleomorphic rhabdomyosarcomas at a much lower frequency than the ARMS seen in Myf6Cre/Wt Pax3P3Fm/P3Fmp53F2-10/F2-10 mice (P < 0.001, log-rank test). These findings suggest that the tumors from Myf6Cre/Wt Pax3P3Fm/P3Fmp53F2-10/F2-10 mice are not caused solely by p53 mutation but by cooperating effects of Pax3:Fkh and p53 mutation.

Conditional mouse models of ARMS share the aggressive features of the human disease. The sites of tumors and stages at necropsy are summarized in Supplementary Tables 7A and 7B. Both Pax3:Fkh, p53 and Pax3:Fkh, Cdkn2a mice developed advanced ARMS tumors, although the frequency of distant hematogenous metastasis in Pax3:Fkh, p53 model was significantly higher than in Pax3:Fkh, Cdkn2a model (χ² test, P < 0.0001). These conditional mouse models showed a predisposition to rapid disease progression, including rapid local tumor growth and invasion, regional lymph node involvement, and distant hematogenous metastasis (Fig. 2A–D; Supplementary Table S7B). For the latter, micro-CT scan showed both macrometastases and alveolar macrophages associated with micrometastases (Fig. 2C and D).

**Transcriptional activation of Pax3:Fkh is associated with ARMS progression.** Although expression of Pax3:Fkh fusion gene is driven by Pax3 promoter in both human ARMS and in our conditional mouse models, promoter activity of Pax3 is predicted to be low in mature myofibers (19). To monitor expression level of Pax3:Fkh during disease progression, we performed qRT-PCR of Pax3:Fkh in adult skeletal muscles from wild-type (WT) and Myf6Cre/Wt Pax3P3Fm/P3Fmp53F2-10/F2-10 mice, as well as primary and metastatic ARMS tumors from Myf6Cre/Wt Pax3P3Fm/P3Fmp53F2-10/F2-10 mice (Fig. 3A). Samples are detailed in Supplementary Table S2. As expected, expression of Pax3:Fkh in Myf6Cre/Wt Pax3P3Fm/P3Fmp53F2-10/F2-10 preeoplastic skeletal muscle was low, whereas Pax3:Fkh expression was >100-fold higher in ARMS tumors. Metastatic tumors expressed Pax3:Fkh at incrementally higher levels than the primary tumors. Pax3:Fkh in the mouse tumor tissues were also detected at protein level using anti-Fkh antibody (Fig. 3B). We also performed immunofluorescent analysis of corresponding tissue samples using anti-GFP antibody as an in situ correlate of Pax3:Fkh expression (Fig. 3C). In our mice, Pax3:Fkh is followed by an internal ribosomal entry site and the eYFP gene; therefore, eYFP expression corresponds to transcriptional activation of Pax3:Fkh in these tissues (5). Whereas eYFP was undetectable in WT and preeoplastic adolescent skeletal muscle, primary and metastatic ARMS tumors strongly expressed eYFP. Expression pattern of eYFP in primary tumors was heterogeneous compared with the uniform expression pattern in metastatic tumors; quantitatively, the number of eYFP-expressing cells in metastatic ARMS tumors was higher than primary tumors (90% versus 33%, P < 0.001). These results suggest that the level of the transcriptional activation of Pax3:Fkh is linked to tumor development and progression and that cooperative cellular events are required in the transformation from preeoplasia to tumor to activate Pax3:Fkh transcription. Later, higher quantitative Pax3:Fkh levels by RT-PCR for metastatic tumor lesions seems to be attributable to the more uniform expression of Pax3:Fkh in tumor cells, but not necessarily higher expression in any individual cell.

**Cross-species comparison of molecular signatures validates the mouse model as representative of human ARMS.** GSEA is a computational method, which has been successfully used to assess whether pathways are conserved between zebrafish and human rhabdomyosarcoma (20, 21). For our GSEA, we tested whether the gene sets up-regulated in mouse ARMS are enriched in human ARMS when compared with other mesenchymal malignancies. The differentially expressed genes were selected by comparing mouse ARMS to 4-week-old WT skeletal muscle at P value of <0.01. Using published database of human sarcomas (13), we performed GSEA with up-regulated gene sets of mouse ARMS (list in Supplementary Table S8). The gene set up-regulated in mouse ARMS was enriched most significantly in human ARMS among all human mesenchymal malignancies (normalized enrichment score = 2.0720, FDR qval < 0.001; Supplementary Table S9). ERMS scored lower (normalized enrichment score = 1.5773, FDR qval = 0.0038). Additional GSEA results using a human rhabdomyosarcoma data set (9) are given in Supplementary Fig. S2. Tamayo and colleagues recently developed a metagene projection methodology to enable a direct cross-species and cross-platform comparison (22). This method can be used to assess the degree to which mouse ARMS displays a transcriptional profile comparable with other human tumors (15). For the purpose of further investigating whether mouse ARMS shares genetic features of human ARMS, metagene projection analysis was undertaken. To define a metagene for human ARMS compared with other human tumors, we used previously published data sets of human mesenchymal tumors (13) and pediatric tumors (14). Although a metagene was defined for each human malignancy, projected
clustering could not entirely separate human ARMS and ERMS (Supplementary Fig. S3A). In addition to our six cases of mouse ARMS samples, published mouse osteosarcoma samples (15) and mouse medulloblastoma samples (16) were used as testing samples. The projected clustering of mouse tumors showed mouse ARMS cluster with human rhabdomyosarcomas (both ARMS and ERMS) and mouse osteosarcomas and medulloblastomas cluster with their human counterparts. Another metagene analysis was performed using a well-characterized data set of human rhabdomyosarcoma (9). Again, however, a defined metagene failed to separate human ARMS and human ERMS completely; instead, 7 of 22 human ARMS clustered with human ERMS (Supplementary Fig. S3B). Hierarchical clustering after metagene projection showed that mouse ARMS cluster with those seven cases of human ARMS, which confirmed that mouse ARMS recapitulates, at least, a subset of human ARMS cases.

The previous literature (8, 9, 23) has identified a subset of genes, which are specifically overexpressed in human ARMS compared with ERMS. Lae and colleagues (9) compared those gene sets and identified 11 genes that are shared in all of those three publications. To further validate that the mouse tumors share the genetic features of human ARMS, expression of those 11 genes, as well as Mycn, another representative alveolar specific gene, were examined by qRT-PCR (Fig. 4) among those 12 genes,

Figure 1. High penetrance of conditional mouse model of ARMS. Biallelic activation of Pax3:Fkhr expression concurrent with p53 or Cdkn2a inactivation is critical for ARMS development. Disease-free survival of Pax3:Fkhr, p53 mouse (A) and Pax3:Fkhr, Cdkn2a mouse (B). P, conditional Pax3:Fkhr allele; F, floxed conditional knockout allele; WT, WT allele. C, comparison of Myf6ICNm/WTPax3P3Fm/P3FmCdkn2aF2-3/F2-3 mice (Pax3(P/P) CDK(F/F)) and Myf6ICNm/WTPax3P3Fm/P3FmCdkn2aF2-3/F2-3 mice (Pax3(P/P) p53(F/F)). For cohort size, see Supplementary Table S6. D, H&E staining (top), trichrome staining (second row), immunohistochemistry positive for Myogenin (third row), and MyoD (bottom) in Pax3:Fkhr, p53, and Pax3:Fkhr, Cdkn2a mouse tumors.
nine genes (Ass1, Cnr1, Dcx, Ela1, Foxf1a, Pipox, Tcfap2b, Wscd1, and Mycn) were significantly overexpressed compared with skeletal muscle. Thus, collectively, mouse ARMS tumors share a common core expression profile with human ARMS tumors.

Cross-species comparison of mouse and human Pax3:Fkhr–positive ARMS identifies a conserved expression signature of ARMS and permits Pax3:Fkhr target identification. To identify a conserved molecular profile of ARMS across species, genes differentially expressed in mouse tumors compared with WT skeletal muscle were projected into human rhabdomyosarcoma versus skeletal muscle. For the human data, published data sets of human young, old, and pathologic skeletal muscle (10, 11) and human rhabdomyosarcoma (8) were used. Genes (1,624) were differentially expressed in mouse ARMS versus skeletal muscle (673 up-regulated genes and 951 down-regulated genes in mouse ARMS; Supplementary Table S8). Among those 1,624 genes, 1,046 genes (392 of 673 up-regulated genes and 654 of 951 down-regulated genes) were also differentially expressed in human ARMS (P < 0.01 by t test in tumors of both species compared with skeletal muscle; Supplementary Fig. S4A). This list may be a mixture of tumor-related and non–tumor-related genes, especially knowing that in vivo studies have shown that Pax3:Fkhr can not only cause tumors but also lead to abnormally developed, disordered (dystrophic) muscle (7, 24). Therefore, we sought to enrich for tumor-specific genes by excluding genes differentially expressed by diseased muscle, thus highlighting 368 genes in the cross-species molecular profile of ARMS (158 up-regulated and 210 down-regulated genes; Supplementary Table S8).

Using a different approach, we went on to identify a Pax3:Fkhr molecular signature conserved across species by combining the 1,624 mouse genes differentially expressed between mouse ARMS with the set of human Pax3:Fkhr–positive ARMS versus fusion-negative ARMS (P < 0.01; Supplementary Fig. S3B; gene list in Supplementary Table S10). Fifty-six intersecting genes were identified, which may be Pax3:Fkhr direct or indirect transcriptional targets.

Among those genes was SKP2, whose expression has been reported to be up-regulated by Pax3:Fkhr (25). The overexpression of SKP2 gene in both mouse and human fusion-positive rhabdomyosarcoma was confirmed by qRT-PCR (Supplementary Fig. S3C). To determine whether SKP2 transcription is regulated by Pax3:Fkhr, NIH3T3 cells were infected with a retrovirus carrying Pax3:Fkhr and then treated with cycloheximide for up to 8 hours (Fig. 5A). Treatment with cycloheximide did not affect SKP2 levels during this time course, suggesting that SKP2 transcription may be directly regulated by Pax3:Fkhr. To further study whether SKP2 is a direct transcriptional target of Pax3:Fkhr, a reporter assay was performed using the SKP2 promoter (Fig. 5B). Serially deleted genomic fragments from the SKP2 promoter region (spanning the 3723bp fragment 5’ upstream of SKP2) were tested for the response to Pax3:Fkhr overexpression in NIH3T3 cells. However, the SKP2 promoter fragments did not show a transcriptional response to Pax3:Fkhr, although the SKP2 promoter did respond to E2F1, a known direct transcription activator of SKP2 gene (Fig. 5B; ref. 17).

Whereas the proximal 3.7kb SKP2 promoter had no activity in response to Pax3:Fkhr, we speculated that Pax3:Fkhr may be up-regulating SKP2 through another cis-element. In keeping with this hypothesis, Barber and colleagues reported from a chromatin immunoprecipitation screen that Pax3:Fkhr can bind to a 220-bp genomic fragment, which is 49 kb downstream (5’) to the SKP2 gene transcription initiation site (Supplementary Fig. S5; ref. 26). The distance, albeit long, is not unprecedented for genes involved in myogenic programming (27). This potential cis-element is conserved across species (Supplementary Fig. S5A). A reporter assay using this 220-bp genomic fragment showed increased luciferase activity when NIH3T3 cells or p53-deficient MEFs were cotransfected with Pax3:Fkhr, and like the PDGFRα reporter control (18), p53 may antagonize Pax3:Fkhr–mediated transcriptional activation of the SKP2 cis-element, depending upon the cellular context (antagonism was seen in NIH3T3 cells, but not in p53-deficient MEFs; Fig. 5C). Thus, this cis-element may be in at least one site by which Pax3:Fkhr regulates SKP2. A definite link between this Pax3:Fkhr responsive element and transcription of the SKP2 gene will likely require future generation of new transgenic animals.

To determine the relevance of SKP2 up-regulation by Pax3:Fkhr, we performed functional studies in human ARMS cells. SKP2 has been reported to be involved in the cell cycle–dependent control of p27Kip1 ubiquitination and, thus, cell cycle entry/tumor cell growth.

Figure 2. The conditional mouse model of ARMS reflects the aggressiveness of the human disease. A, limb tumor upon diagnosis (left) and at 6 d later (right). B, micro-CT scan of a right chest tumor (T). Yellow arrow, lymphatic metastasis; PA, posterior-anterior. C, pulmonary metastases can be quantified using micro-CT–based virtual histology. An 8-μm resolution scan of normal lung (left) and lung with metastasis (right). White arrows, gross metastases (0.8 × 0.92 × 0.74 mm). D, comparison of scan (top left) and histology (top right). Histologic verification was performed after epoxy embedding. Bottom, higher magnification. Yellow arrows point to macrophages found in locations of black-appearing dots on the micro-CT scan. Macrophages are completely surrounded by tumor cells.
To determine whether SKP2 repression can affect cell growth, the human ARMS cell line Rh30 was stably transfected with SKP2-specific short hairpin RNA (shRNA), as described previously (ref. 28; Fig. 5D). Increased protein level of p27kip1, as well as reduced expression of SKP2, was confirmed in SKP2-shRNA cells by Western blotting. Rh30 cells infected with SKP2-shRNA showed substantially reduced cell growth compared with control-shRNA cells. This effect was also confirmed in mouse ARMS cells, derived from a Myf6ICNm/WTPax3P3Fm/P3Fmp53F2-10/F2-10 tumor (Supplementary Fig. S5B). Collectively, these data indicate that SKP2 is a potential transcriptional target of Pax3:Fkhr via a 3′ cis-element and that SKP2 plays a major role in the cell proliferation of ARMS. More broadly, these results suggest that the mouse model of ARMS can serve to identify a Pax3:Fkhr molecular signature and Pax3:Fkhr target genes conserved across species.

The mouse model represents a system for evaluating kinase inhibitors for ARMS. This mouse model was previously used to validate a receptor tyrosine kinase, PDGFRα, as a direct transcriptional target of Pax3:Fkhr and therapeutic target (18). To identify other potential druggable targets in ARMS, we selected a subset of protein kinase genes that were up-regulated in both mouse and human ARMS tumors (Fig. 6A). Among 19 protein kinases up-regulated in mouse tumors, up-regulation of 16 kinases was conserved in human ARMS. From this set, kinase inhibitors are available against seven genes, including VRK1, AURKB, PLK2, PLK4, CDK4, CHEK1, and TK1 (29–31). Overexpression of these kinases was confirmed by qRT-PCR in a larger set of mouse tumors (Fig. 6B). These results validate the future use of this mouse model as a preclinical tool for the study of therapeutic kinase inhibitor strategies in ARMS.
**Discussion**

In this paper, we present a cross-species validation of a genetically engineered mouse model of ARMS. The implicit advantage of using conditional genetic models for preclinical therapeutic testing are that tumors arise in an authentic microenvironment, i.e., skeletal muscle, and that the immune system is intact. The latter may be especially important for the promising cadre of monoclonal antibodies, for which antibody-dependent cellular cytotoxicity may require immunocompetence (32).

Our study shows that this ARMS model is advantageous for preclinical therapeutics for several reasons. We show that the Pax3:Fkhr, p53 model has 100% penetrance by 150 days (young adulthood in a mouse) with a spectrum of disease sites that are comparable with human rhabdomyosarcoma. Histology and immunohistochemical markers also mimic the human disease, as reported here and previously (5, 7). Furthermore, the progression of disease in terms of primary tumor growth and extent of disease are as rapid as or more rapid than the human disease, making the model useful for understanding the underlying disease mechanisms that allow unresectable or metastatic rhabdomyosarcoma to elude therapy.

We show at a cellular level that cooperative factors other than the Pax3:Fkhr fusion or p53 inactivation are likely to be responsible for Pax3:Fkhr transcriptional regulation in preneoplastic muscle. However, once the primary tumor has formed, tumor cells that metastasize appear to be under selection for Pax3:Fkhr expression. Whereas targeting transcription factors, such as Pax3:Fkhr, is therapeutically challenging, one can hope that cooperative factors that facilitate high Pax3:Fkhr transcription might include cell surface receptors or proteins sensitive to small molecule inhibitors. The identification of these cooperative factors that modulate Pax3:Fkhr expression is the subject of ongoing studies.

To validate our model on a whole-genome basis, we performed a cross-species gene expression analysis. Gene set enrichment analysis confirmed that our model is most related to human ARMS among a variety of human sarcomas. We also performed metagene projection. This powerful method of cross-species,
cross-platform analysis (22) has been used recently to compare mouse and human pediatric cancer models among a variety of cancer subtypes. However, this method warrants some caution because results are dependent upon a training set with a large homogeneous collection of each tumor subtype. For rhabdomyosarcomas, which are relatively rare, sample size has been problematic in other studies (15). Nevertheless, we were able to show that, compared with other pediatric cancers, our mouse model is most similar to human rhabdomyosarcomas and specifically human ARMS. We found, however, that despite using the best available microarray data set for rhabdomyosarcoma subtypes, metagene analysis could no better separate human Pax3:Fkhrl-positive ARMS from ERMS than the original report for this data set (9). This result may be due to a technical limitation of this approach and a small sample size or may suggest that ARMS and ERMS (as defined by histology) may be a continuous spectrum of disease. This later possibility, taken in a positive light, suggests that rhabdomyosarcomas might still be further subclassified on

Figure 5. The mouse model phenocopies the human disease, allowing Pax3:Fkhrl target identification. A, Northern blotting showed induction of SKP2 expression by Pax3:Fkhrl was maintained even after cycloheximide treatment. B, reporter assay showing a genomic fragment of 3,723 bp upstream to SKP2 gene did not respond to Pax3:Fkhrl. C, a putative Pax3:Fkhrl binding site at 49 kb downstream (3¶) to SKP2 showed response to Pax3:Fkhrl in NIH3T3 cells and p53-deficient MEFs. PDGFRA promoter was used as a positive control. *, P < 0.01; N.S., no significant difference. D, SKP2 down-regulation represses cell proliferation in human rhabdomyosarcoma cells. Rh30 was stably transfected with SKP2-specific or control shRNA vector. Western blot analysis confirmed reduced steady-state level of SKP2 protein without affecting PAX3-FKHR protein level. Concomitant to reduced SKP2 expression was an increase in p27 protein. Rhabdomyosarcoma cells transfected with SKP2-specific shRNA showed substantially decreased cell growth compared with control nonspecific shRNA (analysis of covariance, P < 0.001). Population doubling time for SKP2-shRNA and nontargeting shRNA was 46.2 and 36.2 h, respectively.
Figure 6. Cross-species identification of overexpressed protein kinases as potential therapeutic targets. A, expression pattern of 19 protein kinases overexpressed in mouse ARMS. Sixteen kinases were significantly up-regulated in human ARMS. Red-highlighted genes are potentially druggable targets. B, qRT-PCR confirming overexpression of the protein kinases for which kinase inhibitors are available.
For all of the strengths of this five allele genetically engineered model (more alleles if you include reporter genes for noninvasive imaging), significant infrastructure investments are required to maintain this disease model system. Because tumors can arise from deep sites, specialized small animal imaging technology is necessary (42) because traditional measurement with calipers at the skin surface nearly always underestimates the extent of disease. Luciferase has been suprisingly noninformative in our model system because tumors have a tendency to be centrally hypovascular and hypoxic (42), thereby unable to have access to the oxygen required by luciferase. The financial investment in maintaining mouse stock lines, husbandry, and genotyping is also nontrivial; therefore, alternative models, such as very successful rhabdomyosarcoma xenograft systems (43) and a recently reported ectopic allograft model (44), are warranted options to our transgenic model. In some instances, certain targets identified from human tumors are not expressed in the cell lines used for xenografts (18). In these cases, the genetically engineered model may not only be essential but also extremely productive. Our laboratory recently identified PDGFRα as a potential therapeutic target from the study of the ARMS preclinical model we report here (18). To follow this example and to make our model more practical for widespread use, we will be soon participating in the National Cancer Institute Pediatric Preclinical Testing Program (45, 46), with the intent of examining efficacy of novel targeted therapies. We will also be providing preclinical testing for outside investigators on a high-volume, low-cost basis. In this cooperative framework, the outlook for new therapies in ARMS may be significantly improved.

Disclosure of Potential Conflicts of Interest

C. Keller: Ownership interest and consultant/advisory board, Namira Biosciences. The other authors disclosed no potential conflicts of interest.

Acknowledgments

Received 9/24/08; revised 12/30/08; accepted 1/8/09.

Grant support: Bradley J. Breidinger Memorial research award from Sarcoma Foundation of America (C. Keller), NIH grant CA074907 (C. Wang), NIH grant CA64202 (F.G. Barr), Alex’s Lemonade Stand Foundation grant (K. Nishijo, and Scott Carter), Foundation grant (C. Keller and K. Nishijo), C. Keller is a member of the Clinical Trial Research Center (PBRCA54174).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Dr. Peter Houghton for graciously providing Rh30 and Drs. Louis Kunkel and Peter B. Kang for the data sets and their kind review of this manuscript.

References


We went on to identify 368 tumor-specific genes in common between mouse and human tumors that could neither be explained as being related to a normal muscle or degenerative muscle phenotype. Next, we used this genetic model to identify potential downstream targets of Pax3/Fkhr. Identifying Pax3/Fkhr targets has been the subject of numerous antecedent studies using many different valid approaches (i.e., transfection of rhabdomyosarcoma or nonrhabdomyosarcoma cells with Pax3/Fkhr, comparison between primary tumors, or combinations thereof; refs. 23, 26, 32–37). Our approach is meant only to be complementary. In the end, primary human tumor samples (Pax3/Fkhr–positive versus fusion-negative ARMS) are the definitive study set for such determinations, but getting large numbers of high-quality rapidly processed samples of these rare tumors has been a challenge for the field. Nevertheless, our cross-species approach identified 56 candidate target genes of Pax3/Fkhr, including SKP2 (Fig. 5A, right). SKP2 has been suggested to be a target gene of Pax3/Fkhr, but not Pax5, in fibroblasts (25). We have extended this result by validating SKP2 as a Pax3/Fkhr target in vivo. SKP2 is a component of the SCF (SKP1-CUL1-F-box) protein complex that mediates the ubiquitination and proteasomal degradation of cell cycle regulatory genes, including p27 (25, 38), thereby accelerating cell cycle progression. Ironically, SKP2 also interacts with and promotes the ubiquitin-mediated degradation of Fkhr (FoxO1A; ref. 39). This SKP2-mediated degradation of Fkhr requires phosphorylation of Fkhr at Ser-256 (39), which is in fact retained by Pax3/Fkhr (40). Interestingly, Fkhr Ser-256 phosphorylation also reduces binding of Fkhr to DNA and causes nuclear exclusion of Fkhr when Thr-24 and Ser-319 are also phosphorylated (41). The extent to which the phosphorylation of this serine residue in Pax3/Fkhr can be enforced and Ser-319 are also phosphorylated (41). The extent to which the phosphorylation of this serine residue in Pax3/Fkhr can be enforced to take advantage of SKP2 overexpression, SCF-mediated degradation, and Pax3/Fkhr nuclear exclusion is the topic of the ongoing investigation.

To identify new therapeutic targets, we examined the expression of potentially “druggable” kinases. The range of available kinase inhibitors is growing rapidly; therefore, we examined the cross-species rhabdomyosarcoma expression of kinases known to have an inhibitor available preclinically or clinically. We identified seven kinases, including an aurora kinase and two polo-like kinases.


Credentialeding a Preclinical Mouse Model of Alveolar Rhabdomyosarcoma

Koichi Nishijo, Qing-Rong Chen, Lei Zhang, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/69/7/2902

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2009/04/15/69.7.2902.DC1

Cited articles
This article cites 44 articles, 24 of which you can access for free at:
http://cancerres.aacrjournals.org/content/69/7/2902.full.html#ref-list-1

Citing articles
This article has been cited by 12 HighWire-hosted articles. Access the articles at:
/content/69/7/2902.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.